Application No.: 09/531,851
Attorney Docket No.: 03678.0028.US04

REMARKS

Applicants thank Examiner Owens for the telephone interviews dated March 17 and 19, 2004. In the telephone interview, the Examiner requests that Applicants submit evidence that dinucleoside polyphosphates are more stable than mononucleotides, for support of the arguments submitted in the Response dated December 18, 2003.

Applicants are submitting herewith Luthje, *et al.* (Eur. J. Biochem. 173: 241 (1988)). At page 245, left column, lines 10-12 from the bottom, the reference describes "In contrast to ATP and ADP, which are rapidly degraded by ectonucleotidase present on blood cells and on the endothelial lining, the dinucleotides are only slowly degraded."

Applicants are also submitting herewith a poster publication (Shaver, *et al*), which was presented at XIVth World Congress of Pharmacology, San Francisco, CA, July 7-12, 2002. In the poster presentation, a series of synthetic dinucleotides were examined for their relative stabilities on bronchial tissue yielding a rank order of $dCp_4U = Cp_4U > dAp_4U > Cp_4C > Ip_4U > dGp_4U > Up_4U = Xp_4U > Ap_4A >> UTP$.

Both of the above two references show that dinucleoside polyphosphates are more stable than mononucleotides.

Applicants respectfully remind the Examiner that Applicants have submitted a Request for Interference with Patent (U.S. Patent No. 6,254,188) on November 28, 2001.

Respectfully submitted,

Date: March 19, 2004

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Eur. J. Biochem. 173, 241 – 245 (1988)
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Catabolism of Ap_4A and Ap_3A in whole blood

The dinucleotides are long-lived signal molecules in the blood ending up as intracellular ATP in the erythrocytes

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(Received September 29/December 23, 1987) – EJB 87 1091

Adenosine(5')tetraphospho(5')adenosine (Ap_4A) and adenosine(5')triphospho(5')adenosine (Ap_3A) are stored in large amounts in human platelets. After activation of the platelets both dinucleotides are released into the extracellular milieu where they play a role in the modulation of platelet aggregation and also in the regulation of the vasotone. It has recently been shown that the dinucleotides are degraded by enzymes present in the plasma [Lüthje, J. & Ogilvie, A. (1987) *Eur. J. Biochem. 169*, 385–388]. The further metabolism as well as the role of blood cells has not been established. The dinucleotides were first degraded by plasma phosphodiesterases yielding ATP (ADP) plus AMP as products which were then metabolized to adenosine and inosine. The nucleosides did not accumulate but were very rapidly salvaged by erythrocytes yielding intracellular ATP as the main product.

Although lysates of platelets, leucocytes and red blood cells contained large amounts of Ap_3A-degrading and Ap_4A-degrading activities, these activities were not detectable in suspensions of intact cells suggesting the lack of dinucleotide-hydrolyzing ectoenzymes. Compared to ATP, which is rapidly degraded by ectoenzymes present on blood cells, the half-life of Ap_4A was two to three times longer.

Since the dinucleotides are secreted together with ADP and ATP from the platelets, we tested the influence of ATP on the rate of degradation of Ap_4A. ATP at concentrations present during platelet aggregation strongly inhibited the degradation of Ap_4A in whole blood. It is suggested that *in vivo* the dinucleotides are protected from degradation immediately after their release. They may thus survive for rather long times and may act as signals even at sites far away from the platelet aggregate.

Adenosine(5')tetraphospho(5')adenosine (Ap_4A) and the homologue adenosine(5')triphospho(5')adenosine (Ap_3A) are stored in large amounts in human platelets [1, 2]. Both dinucleotides are released from the platelet dense granules into the extracellular milieu after activation of the cells [1, 2]. Ap_3A and Ap_4A have been suggested to play a role in platelet physiology. Ap_4A inhibits ADP-induced platelet aggregation in platelet-rich plasma as well as in whole blood [3, 4]. In contrast, Ap_3A causes a gradual aggregation of platelets. This Ap_3A-induced aggregation is mediated by an enzymatic activity in plasma that hydrolyzes Ap_3A, producing ADP, a potent stimulator of platelet aggregation [4].

Furthermore, previous results have shown that Ap_4A and Ap_3A not only modulate platelet aggregation but also have vasomotor effects [5]. Thus, increasing experimental evidence suggests that these dinucleotides are regulatory molecules involved in the complex process of hemostasis.

With respect to their possible role as extracellular signals it is of interest to know how long the dinucleotides can survive in the blood stream. After their release from the platelets the lifetime of Ap_3A and Ap_4A in the blood depends on the catabolic capacities of the plasma and the intact blood cells. In human plasma three enzymes that split the dinucleotides have been described [6]. The enzymes cleave the molecules asymmetrically, always yielding AMP as one product. The predominant activity, comprising about 95% of the total activity, has been purified and characterized biochemically [7]. However, it is hitherto not known whether ectoenzymes of blood cells are involved in the metabolism of Ap_4A and Ap_3A. Ectoenzymes splitting ADP and ATP are well known to be present on several blood cells such as erythrocytes [8], leukocytes [9, 10] and platelets [11–13]. In addition to the enzymatic capacities of the blood, other factors, such as competetive substrates, may influence the lifetime of the dinucleotides. Since Ap_3A and Ap_4A are released from the platelets along with other storage pool nucleotides, mainly ADP and ATP [2], it is conceivable that these compounds might affect the degradation rate of the dinucleotides in the blood. We here present data showing that ATP at concentrations present during platelet aggregation can profoundly affect the rate of hydrolysis of Ap_4A in whole blood.

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Abbreviations. Ap_3A, adenosine(5')triphospho(5')adenosine; Ap_4A, adenosine(5')tetraphospho(5')adenosine; PEI-cellulose, poly(ethyleneimine)-cellulose.

Enzymes. The enzymes in the plasma splitting Ap_3A and Ap_4A are 5'-nucleotide phosphodiesterases (EC 3.1.4.1); lactate dehydrogenase (EC 1.1.1.27).

MATERIALS AND METHODS

Reagents

[2,8-^{3}H]Ap_4A (TRQ 4405; 4.3 Ci/mmol) and [2,8-^{3}H]ATP (TRK 622; 36 Ci/mmol) were purchased from Amersham.

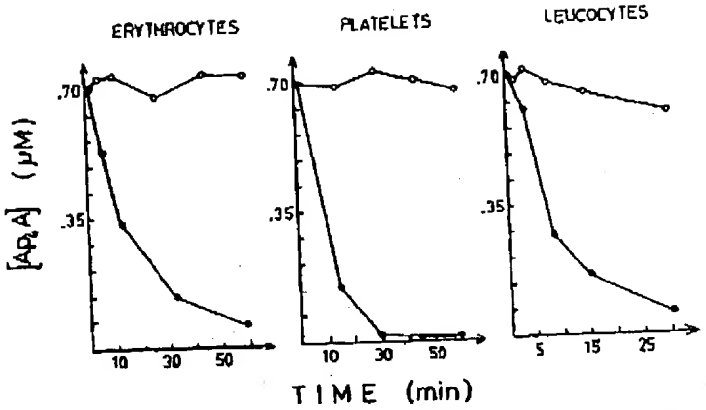

Fig. 1. *Degradation of [^{3}H]Ap$_4$A by washed human blood cells and blood cell lysates.* Suspensions of intact blood cells (O——O) or Triton X-100 lysates of blood cells (●——●) were incubated with Ap$_4$A (0.7 μM). Degradation of Ap$_4$A was followed by a thin-layer technique as described under Materials and Methods. Cell numbers of the suspensions and of the lysates: erythrocytes 4×10^9/ml, platelets 1.3×10^9/ml, leucocytes 3.9×10^7/ml

Analysis by thin-layer chromatography revealed a purity of over 97%. Ap$_4$A, ATP, Ap$_3$A, dextran and prostaglandin E$_1$ were from Sigma (Munich, FRG). Triton X-100 and trichloroacetic acid were purchased from Roth (Karlsruhe, FRG). Pyruvate and NADH were obtained from Boehringer (Mannheim, FRG). Salts and thin-layer chromatography plastic sheets (PEI-cellulose F) were from Merck (Darmstadt, FRG). Blood was obtained from healthy laboratory volunteers.

Dextran was used as a 6% (mass/vol.) solution in 0.15 M NaCl. The isotonic buffer A contained 95 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM glucose, 45 mM Tris/HCl (pH 7.4) and 0.5 g/l bovine serum albumin. Buffer B was as A except for a higher content of $MgCl_2$ (6 mM).

Isolation of blood cells

Fresh human blood was anticoagulated with heparin (5 U/ml final concentration). For preparation of platelets, blood was centrifuged at $150 \times g$ for 15 min at room temperature. The platelet-rich plasma was completely sucked off except for the upper 3 mm above the buffy coat in order to avoid contamination with leukocytes. Platelet-rich plasma was mixed with prostaglandin E$_1$ (3 μM final concentration) to prevent clumping of platelets during centrifugation. Prostaglandin E$_1$ had been confirmed in separate experiments to have no influence on the results. Platelets were pelleted by centrifugation at $1700 \times g$ for 5 min, washed twice with buffer A and finally suspended in buffer B.

Red blood cells were prepared from the pellet obtained during the preparation of platelet-rich plasma. The buffy coat and the upper part of the red pellet were sucked off and discarded. The remaining erythrocytes were suspended and washed five times in buffer A. After the last washing the cells were suspended in buffer B.

Leucocytes were prepared by dextran sedimentation. 40 ml blood were mixed with 8 ml dextran solution. After sedimentation for 60 min at room temperature the supernatant was centrifuged at $150 \times g$ for 12 min. The pellet was treated with 0.87% (mass/vol.) NH_4Cl to lyse residual erythrocytes. Then the leucocytes were washed three times with buffer A and after that resuspended in buffer B. The cell count was determined under the microscope in a Neubauer chamber.

Determination of the Ap$_4$Aase (Ap$_3$Aase) activity

a) Cell suspensions and cell lysates. Cell suspensions were mixed with labelled Ap$_4$A (0.7 μM final concentration) and incubated at 25°C with gentle shaking. The total volume of the assay was 0.8 ml. At various times aliquots of 80 μl were taken and immediately mixed with 16 μl stop solution containing 50 mM Na_2EDTA and 5 mM Ap$_4$A in ice-cold physiological saline. The samples were centrifuged to remove the cells and then the supernatants were treated with an equal volume of ice-cold 20% trichloroacetic acid. Extraction of nucleotides and neutralization of the extracts were performed as described [14]. Samples of 4 μl of the neutralized extracts were spotted onto a PEI-cellulose thin-layer sheet. After separation of Ap$_4$A from its reaction products by developing the thin layer 1) in H_2O and 2) in 0.85 M LiCl, radioactivity in the nucleotide spots was determined as described [7]. Ap$_3$Aase activity was measured by incubating cell suspensions with unlabelled Ap$_3$A (0.7 μM final concentration) under identical conditions. The procedure was analogous to that for Ap$_4$A with the exception that stopping of the reaction by mixing aliquots with nucleotide and EDTA was omitted. Instead aliquots were briefly centrifuged and the supernatants were immediately extracted with trichloroacetic acid. Ap$_3$A in the neutralized extracts was determined with a coupled bioluminescence assay [15]. Cell lysates were prepared by adding Triton X-100 to the cell suspensions (0.5% final concentration). Enzyme activities in lysates were determined as described for cell suspensions.

b) Whole blood and plasma. Blood or plasma was incubated with labelled Ap$_4$A or ATP at 0.7 μM under identical conditions to (a). The reactions were stopped by mixing aliquots with ice-cold solutions containing 50 mM Na_2EDTA and 5 mM Ap$_4$A (or 5 mM ATP). After separation of the cells the nucleotides were extracted and neutralized. The following procedure was identical to that described in (a). For extraction of nucleotides from whole blood (including intracellular pools) the reaction was stopped by pipetting aliquots directly into equal volumes of ice-cold 20% trichloroacetic acid.

Determination of lactate dehydrogenase activity

Lactate dehydrogenase activity was determined in blood cell suspensions as a measure for cell damage. These values

were compared with the activities measured in cell lysates which were assumed to correspond to 100% cell damage. The assay contained in a total volume of 1.1 ml: 1.0 ml phosphate buffer (0.1 M, pH 7.4), 25 µl NADH (0.01 M) and 50 µl sample under investigation. The reaction was started with 25 µl pyruvate (0.1 M). The assay was performed at 25°C. The disappearance of NADH at 366 nm was followed using a Shimadzu spectrophotometer coupled to a chart recorder. Enzyme activities were expressed as initial velocities calculated from the recordings.

RESULTS

Experiments with isolated blood cells

As shown in Fig. 1 intact erythrocytes did not exhibit any Ap_4A-degrading activity. In contrast, total lysates of red blood cells contained highly active enzymes which degraded Ap_4A.

Experiments with platelets revealed very weak or, in some experiments, no activities in the suspensions, whereas lysates always exhibited large amounts of Ap_4A-degrading activity (Fig. 1). After centrifugation of the suspension these activities (about 1–2% of the activity in the lysate) were still detectable in the supernatant suggesting that the enzyme was not bound to the platelets. Furthermore, the supernatants contained small amounts (1–2% of the lysate activity) of lactate dehydrogenase activity indicating that a small fraction of the platelets had been damaged and intracellular enzymes had appeared in the suspension medium. Similarly suspensions of leukocytes always contained small amounts of Ap_4A-degrading activity (Fig. 1), but low activities of lactate dehydrogenase were also present.

These experiments were repeated with Ap_3A as a substrate. Unlabelled Ap_3A (0.7 µM final concentration) was used, and its concentration was determined with a coupled bioluminescence assay. The results were very similar to those obtained with Ap_4A (not shown). In conclusion, lysates of red blood cells, platelets and leucocytes contained high activities of Ap_4A-degrading and Ap_3A-degrading enzymes. However, these enzymes were not detectable in suspensions of intact cells suggesting that ectoenzymes splitting the dinucleotides were either not present on blood cells or only in negligible amounts.

Half-lives of Ap_4A and ATP in whole blood and in plasma

The time course of degradation of Ap_4A in whole blood and in plasma is shown in Fig. 2. The half-life in plasma was 2 min, whereas in blood this value was 4.4 min. In serial experiments with blood from different donors these results were confirmed (Table 1). On average the half-life of Ap_4A was 2.9 min in plasma and 5.9 min in whole blood. The longer half-life of Ap_4A in blood could be explained by the hematocrit, which is responsible for the higher initial concentration of Ap_4A in blood (roughly doubled) as compared to plasma. This should markedly prolong the half-life of Ap_4A in blood when taking into account the low K_m (0.6 µM) of the main plasma hydrolase [7]; this consideration also applies for ATP.

For comparison, ATP under identical experimental conditions had a mean half-life of 3.6 min in plasma and 2.2 min in blood. Thus, in contrast to Ap_4A, ATP was degraded faster in blood than in plasma. When ATP was tested at 40 µM, the half-life in blood was 11.5 min whereas in plasma the half-life could not be determined because of the slow degradation. The rather short lifetime of ATP in whole blood suggested that ectoenzymes on blood cells contribute significantly to the catabolism of ATP.

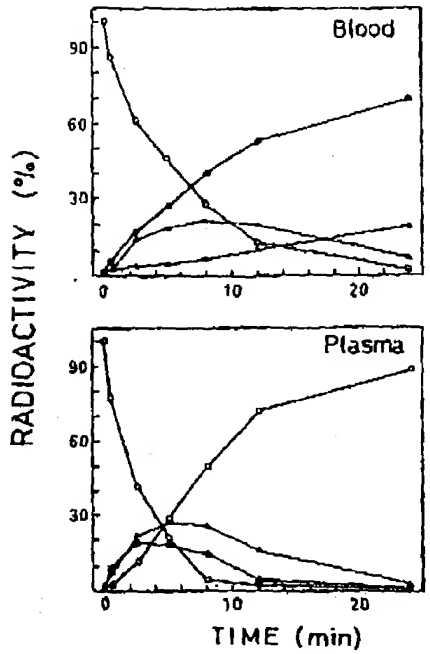

Fig. 2. *Degradation of Ap_4A in human blood and plasma.* Heparinized whole blood or plasma was incubated with labelled Ap_4A at 0.7 µM. At various times aliquots of blood (or plasma) were removed and immediately extracted with trichloroacetic acid. The nucleotides of the neutralized extracts were separated by thin-layer chromatography. The total radioactivity measured in the spots of all the nucleotides mentioned below was always constant and was set as 100%. In whole blood no formation of adenosine and inosine was observed, and in plasma no ADP was detected; for reasons of clarity these data are not shown in the figures. Ap_4A (○——○), ATP (●——●), AMP (△——△), ADP (▲——▲), adenosine plus inosine (□——□). In plasma inosine was detectable after 12 min of incubation

The lifetime of Ap_4A in blood is influenced by ATP

The hitherto single known source of the dinucleotides in human blood is the platelets. After activation of the platelets Ap_3A and Ap_4A are secreted together with ADP and ATP from the dense granules reaching 0.5–1 µM Ap_4A (Ap_3A) and 20–40 µM ATP when assuming a homogeneous distribution in blood [2, 16]. We tested whether ATP at concentrations present during platelet aggregation would affect the rate of hydrolysis of Ap_4A.

Fig. 3 demonstrates that the degradation of Ap_4A in whole blood was almost completely inhibited by ATP at a concentration of 25 µM. This was confirmed by further experiments with blood from several donors. In most experiments a concentration of 25 µM ATP was sufficient to inhibit hydrolysis of Ap_4A totally (not shown). Experiments with various concentrations of ATP revealed that 8–10 µM ATP inhibited the rate of Ap_4A degradation by 50% (Fig. 4). Pyrophosphate, which is also released from the dense granules

Table 1. *Half-lives of Ap_4A and ATP in human blood and plasma*
Radioactively labelled nucleotides were used at a concentration of 0.7 μM. Degradation was measured with a thin-layer chromatography technique as described in Materials and Methods. Values are means ± SD. number of experiments in parentheses

Location	Half-life of	
	Ap_4A	ATP
	min	
Blood	5.88 ± 1.18 (9)	2.21 ± 0.31 (7)
Plasma	2.88 ± 0.54 (8)	3.60 ± 0.85 (5)

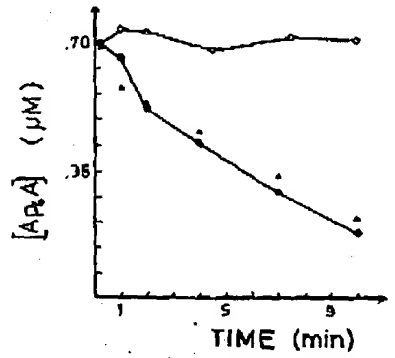

Fig. 3. *Degradation of Ap_4A in human whole blood. Effects of ATP and sodium pyrophosphate.* Whole blood was incubated with tritiated Ap_4A (0.7 μM) in the presence of ATP at 25 μM (O——O), pyrophosphate at 50 μM (▲——▲) or without any addition (●——●). Degradation of Ap_4A was measured with a thin-layer chromatography technique as described in Materials and Methods

in similarly large amounts, compared to ATP and ADP [17, 18], had no effect on the rate of Ap_4A hydrolysis (Fig. 3).

Metabolic fate of the dinucleotides in whole blood

Results of experiments investigating the degradation of labelled Ap_4A and its products in human blood are depicted in Fig. 2. In plasma Ap_4A was cleaved into ATP and AMP and these nucleotides were further metabolized to adenosine and inosine. In contrast, in whole blood the main product was ATP.

From the physiological point of view it is interesting to note that adenosine, which accumulated in plasma from Ap_4A, could not be detected at all in whole blood. Adenosine is a potent inhibitor of platelet aggregation and an effective vasodilator. In whole blood adenosine and inosine are rapidly taken up via the nucleoside transporter of the blood cells [19]. Inside the cells the nucleosides are rephosphorylated [20]. Thus the ATP formed in whole blood corresponded to intracellular ATP (mainly erythrocytes).

This explanation was confirmed by two experiments. First, in the presence of 10 μM dipyridamole, which inhibits the transport of nucleosides through cell membranes [19], the main products of Ap_4A degradation in whole blood after incubation for 20 min at 25°C were adenosine and inosine, and not ATP (not shown). Further degradation of inosine to hypoxanthine by plasma enzymes was not followed [21].

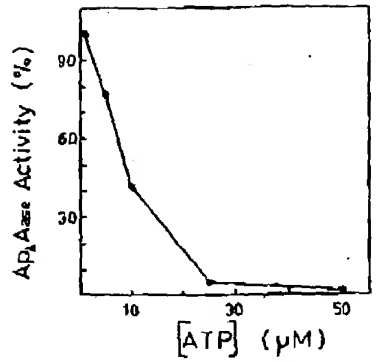

Fig. 4. *Inhibition by ATP of Ap_4A hydrolysis in whole human blood.* Labelled Ap_4A (0.7 μM) was incubated in whole blood in the presence of various concentrations of ATP. Initial velocities were calculated from the kinetics and plotted as function of the ATP concentration. The initial rate of Ap_4A hydrolysis in the absence of ATP was set as 100%

Secondly, when the blood cells were separated from the plasma prior to the extraction with trichloroacetic acid, no ATP was found in the plasma but rather in the blood cells (not shown).

DISCUSSION

This paper deals with the metabolism of the dinucleotides Ap_4A and Ap_3A in unfractionated human blood. In contrast to plasma, which has been shown to contain different Ap_3A-degrading and Ap_4A-degrading enzymes [6, 7], the formed elements of blood do not contribute to the degradation of the dinucleotides. Our results suggest that red blood cells, platelets and leukocytes lack ectoenzymes capable of splitting Ap_4A and Ap_3A. Thus exclusively the enzymes present in the plasma are responsible for the primary degradation of Ap_4A and Ap_3A in the blood. The first step is catalyzed by phosphodiesterases [6], mainly by a glycoprotein complex with a molecular mass of 230 kDa [6, 7]. The resulting nucleotides (ATP, ADP and AMP) are further degraded to adenosine, inosine and finally to hypoxanthine by different enzymes (phosphodiesterases, phosphatases, 5'-nucleotidase, deaminase etc.). In contrast to the first step, which only occurs in the plasma, the subsequent reactions are catalyzed by enzymes present in the plasma as well as by ectoenzymes on blood cells [8–13, 21].

An important role of the blood cells in the metabolism of the dinucleotides, however, is the clearance of adenosine and inosine from the plasma. Adenosine, which is a potent inhibitor of platelet aggregation and an effective vasodilator, does not accumulate in the blood.

The lack of Ap_3A-degrading and Ap_4A-degrading ectoenzymes on blood cells may account for the relatively long lifetime of Ap_4A in whole blood (Table 1). ATP has a markedly shorter half-life in blood. In contrast to the dinucleotides, ATP is known to be degraded by ectoenzymes present on erythrocytes [8], leucocytes [9, 10] and on platelets [11, 12]. Similarly, activities metabolizing ADP have also been detected on various blood cells [8, 13].

When determining the half-life in whole blood of Ap_4A alone at 0.7 μM, a concentration which may occur under physiological conditions [16], values between five and six m

nutes were measured (Table 1). Physiologically, however, Ap_4A is not secreted alone but together with other nucleotides, mainly ADP and ATP, which are released in 20–40-fold excess over the dinucleotides [2]. In the presence of ATP the degradation rate of Ap_4A is dramatically decreased. ATP at 9 μM, i.e. a concentration below that expected to occur *in vivo* after the platelet release reaction, has been shown to inhibit the rate of Ap_4A hydrolysis by 50%. ATP at a concentration of 25 μM almost completely inhibited Ap_4A degradation for more than ten minutes. Thus, it appears that *in vivo* Ap_4A, after its release from platelets, is protected from degradation by ATP (and probably ADP). In molecular terms the effect can be explained by a competitive inhibition of the Ap_4A hydrolase. ATP as well as ADP are strong inhibitors of the hydrolase purified from plasma [7].

The results presented here for Ap_4A probably also apply to Ap_3A since the metabolism of both dinucleotides is analogous. Like Ap_4A, Ap_3A is not metabolized by ectoenzymes on the surface of blood cells, and in plasma both dinucleotides are degraded by the same enzyme [6, 7]. When tested at a concentration of 1 μM the purified hydrolase degrades Ap_4A 2.5-fold faster than Ap_3A, suggesting that in blood Ap_3A will even have a longer lifetime than the homologue Ap_4A [7].

We have considered the metabolism of the dinucleotides in whole blood but the picture will remain incomplete as long as the possible contribution of the endothelium is not taken into account. Endothelial cells line the inner blood vessel walls and contain various ectonucleotidase activities [22]. Very recently Goldman and coworkers have shown that intact porcine aortic endothelial cells can efficiently hydrolyze extracellular Ap_3A. In the presence of ATP the degradation of Ap_3A was strongly inhibited [23]. We have demonstrated that Ap_4A and Ap_3A are degraded by intact bovine vascular endothelial cells, with half-lives of 40–80 min (at 1 μM). For comparison, ATP and ADP under identical conditions were metabolized at least 20 times faster [24]. Thus it appears that Ap_4A and Ap_3A are long-lived molecules in the blood vessel. In contrast to ATP and ADP, which are rapidly degraded by ectonucleotidases present on blood cells and on the endothelial lining, the dinucleotides are only slowly degraded. The regulatory role of ATP, which is released together with the dinucleotides from the platelets, appears to us of especial importance. Besides its antagonistic function in ADP-induced platelet aggregation, ATP strongly inhibits the Ap_4Aase and Ap_3Aase activities in plasma as well as on endothelial cells, enabling the dinucleotides to survive for a longer time. Thus, the dinucleotides can diffuse away from the site of thrombus formation without being destroyed and may act at distant sites as signal molecules. Current evidence implies that Ap_3A and Ap_4A are not only involved in platelet aggregation but also in the modulation of the vasotonus [5].

This work was supported by the *Deutsche Forschungsgemeinschaft*. We gratefully acknowledge the excellent technical assistance of Margot Rehm, the continuous support by Prof. Dr W. Kersten and thank all the donors for supplying us with blood.

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XIV th World Congress of Pharmacology, July 7-12, 2002

PROGRAM No. 146.11

P2Y RECEPTOR AGONISTS: A COMPARISON OF MIXED-BASE DINUCLEOTIDES VERSUS SAME-BASE DINUCLEOTIDES AND THEIR DEOXYRIBO-ANALOGUES

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ABSTRACT

$P2Y_2$ receptors are found in tissues and organ systems throughout the body and are implicated in various physiological responses with the natural ligands being UTP and ATP. Certain symmetrical dinucleotides, such as Up_4U (EC_{50} = 65 nM), are also agonists of this receptor with a greater chemical and metabolic stability than that of the natural ligands. We discovered that mixed-base dinucleotides, such as Cp_4U (EC_{50} = 210 nM) and its deoxyribo analogue dCp_4U (EC_{50} = 280 nM), retained this receptor activity while exhibiting an improved metabolic stability. By HPLC analysis, both analogues are equally stable to hydrolysis by human bronchial epithelia with half-lives approaching 3 hr (UTP $t_{1/2}$ < 5min). A series of synthetic dinucleotides were examined for their relative stabilities on bronchial tissue yielding a rank order of dCp_4U = Cp_4U > dAp_4U > Cp_4C > Ip_4U > dGp_4U > Up_4U = Xp_4U > Ap_4A >> UTP. These more stable $P2Y_2$ agonists may have greater therapeutic utility compared to mononucleotides.

INTRODUCTION

P2 receptors (by IUPHAR convention, formerly termed P2-purinoceptors) are extracellular nucleotide binding receptors, which are divided into P2X ionotropic receptors and P2Y metabotropic receptors. P2Y receptors have seven transmembrane regions and are activated by extracellular nucleotides: $P2Y_2$ and $P2Y_{11}$ are all fully activated by the action of ATP; ADP is a full agonist on $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$; UTP fully activates $P2Y_2$ and $P2Y_4$, and UDP is the natural ligand for $P2Y_6$.

These natural nucleotides, despite their efficacy and potency, have drawbacks as potential therapeutic compounds. The phosphate chain at the 5'-position of the nucleoside is attacked by a variety of ecto-nucleotidases thereby resulting in short *in vivo* half lifes for these compounds. The phosphate moieties also impart a limited chemical stability to these compounds, which dictates refrigerated storage.

Dinucleoside polyphosphates, in which two nucleosides are attached through the 5'-hydroxyls via a phosphate chain, gave much greater chemical stability than the corresponding parent nucleotides and also exhibit a much higher resistance to enzymatic digestion.

Many of these stabilized dinucleotides are potent agonists at various subtypes of P2Y receptors. The effect of nucleobase and sugar configuration on agonist activity and relative stability will be discussed.

MATERIALS & METHODS

TEST COMPOUNDS

UTP, UDP, 2MeSADP, ATP, ADP, were purchased from Sigma. All other test compounds were prepared at Inspire Pharmaceuticals.

CELL LINES

1321N1 human astrocytoma cells stably expressing human recombinant P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$); C6 rat gboma cells (endogenous $P2Y_{12}$) expressing the chimera Gq/i2 ($rP2Y_{12}$) were established at Inspire Pharmaceuticals.

CALCIUM MOBILIZATION ASSAY

1321N1 cells were grown in black wall/clear bottom 96 well cell culture plates. Forty-eight hours after plating, the growth medium was aspirated and replaced with Hank's buffer containing 2.5 µM Fluo-3 AM. Following a 60-minute incubation, cells were washed free of dye and stimulated with the indicated concentrations of P2Y receptor agonists/antagonists. Intracellular calcium mobilization was determined by measuring the changes in fluorescence intensity using the FLIPR (Molecular Devices Corp., Sunnyvale, CA).

DATA ANALYSIS AND STATISTICS

Data are expressed as the mean ± standard error of the mean and were replicated in at least 4 experiments. Curve fitting was performed using PRISM 3.0, (GraphPad Software, San Diego, CA).

SYNTHETIC METHOD

The dinucleotides presented here were prepared according to a published procedure.[2] In general, 1.5 equivalents of the tributylamine salt of a nucleoside triphosphate was reacted with dicyclohexylcarbodiimide in anhydrous DMF for 30 minutes.[2] This solution was extracted twice with hexane then treated with 1 equivalent of the tributylamine salt of the desired monophosphate in DMF at 40-50°C for 8-24 hr. The reaction is worked up by quenching with bicarbonate buffer then purified by preparative anion exchange chromatography using a Hamilton PRP-X100 column, 250x50 mm, 10mm, with a linear gradient from water to 90% 1M NH_4HCO_3/10% acetonitrile. The tributylamine salts of the nucleotides were prepared from the sodium salts by treatment in water with a cationic ionic exchange resin in the hydrogen form, followed by neutralization with an excess of NBu_3, evaporation, and lyophilization.

RESULTS

P2Y Receptor Selectivity of Dinucleoside Polyphosphates

Compound	$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	$P2Y_{12}$
UTP	NR	0.05	0.05	>10	NR
UP_4U	NR	0.065	0.35	14	NR
CP_4U	>100	0.21	0.06	>10	NR
AP_4U	0.27	0.13	0.045	>30	NR
IP_4U	NR	0.20	0.15	>10	NR
IP_4A	0.55	0.17	NR	NR	NT
TP_4U	NR	0.22	0.41	NR	NT
XP_4U	NR	0.11	0.24	5.52	NR
CP_4C	SR	SR	NR	SR	NR
AP_4C	0.75	0.14	NR	>100	NT
AP_4A	0.19	0.05	NR	NR	NR
IP_4I	NR	0.73	SR	>10	NR
XP_4X	NR	1.55	3.58	>10	NT
dCP_4U	NR	0.28	0.68	>10	NR
dCP_4A	0.75	0.15	NR	NR	NT
dAP_4U	0.91	0.75	0.25	0.96	NR
dGP_4U	SR	0.061	0.14	1.68	NR

Table 1: EC_{50} data is an average of at least 2 runs for each compound and is expressed in µM; SR=slight response (>100 µM); NR=no response at 100 µM; NT=not tested.

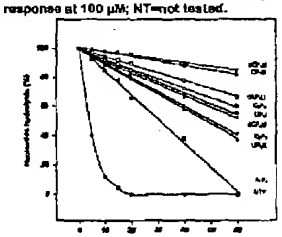

Figure 1. Time-course of the metabolism of various nucleotides by human normal bronchial cells. The cells were grown to confluence on an air-liquid interface and differentiated into a ciliated cell sheath over 4 weeks. The cells were pre-incubated 30 min at 37 °C in Krebs buffer (0.35 ml apical/2 ml basolateral; pH 7.4). The assays were started with 0.1 mM nucleotide added to the apical buffer. Aliquots of 30 ml were transferred to 0.3 ml ice-cold water and boiled during 6 min. Their nucleotide content was analyzed by HPLC. Data are expressed as percent of initial peak (SEM < 10 %; N = 4-8).

RESULTS (CONTINUED)

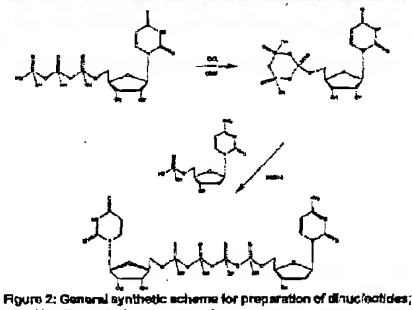

Figure 2: General synthetic scheme for preparation of dinucleotides; dCP_4U is illustrated as an example.

CONCLUSION

- All dinucleotides are more stable on normal human bronchial epithelial cells than mononucleotides.
- In general, symmetrical dinucleotides are less stable on normal human bronchial epithelial cells than are unsymmetrical dinucleotides.
- All of the dinucleotides tested had micromolar or submicromolar activity at $P2Y_2$ receptors except CP_4C.
- CP_4C produced a small or no response at concentrations up to 100 µM in all receptors tested.
- The presence of adenosine in a dinucleotide imparts activity on $P2Y_1$ with the highest potency observed with the symmetrical AP_4A.
- All dinucleotides were either inactive or weak agonists at $P2Y_6$ receptors.
- None of the dinucleotides studied here had agonist activity at $P2Y_{12}$ receptors.

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